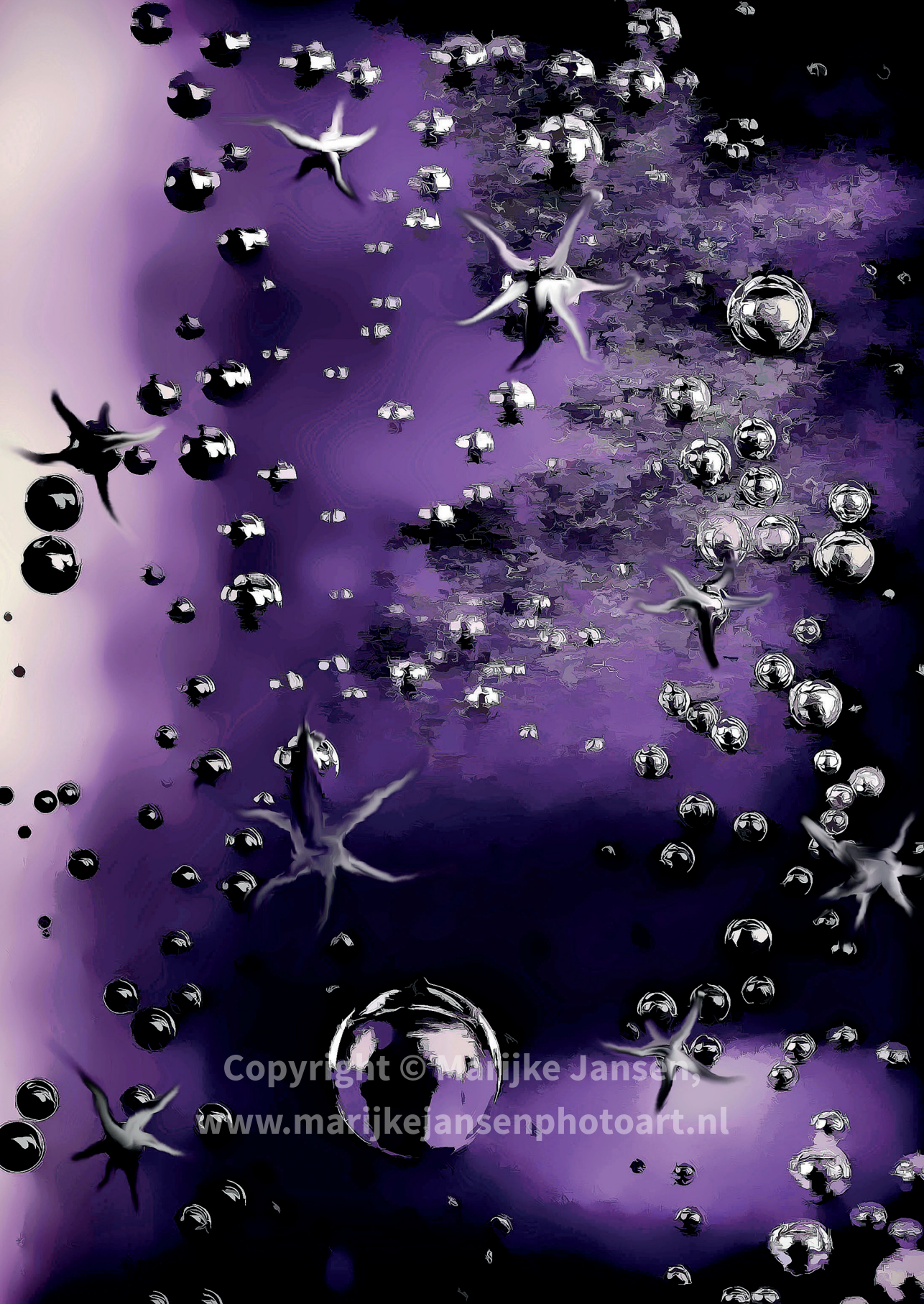

CHAPTER 9

GENERAL DISCUSSION

General Discussion

Viruses have been associated with several neurological disorders. Throughout the years, developments in detection methods and identification of viruses within the central nervous system (CNS), have supported the notion that viruses can infect the brain and spinal cord. Furthermore, these studies have provided insight into possible associations with neurological disease. In this respect, viruses have been demonstrated to be capable of causing acute CNS disorders, including inflammation of the brain, or encephalitis, which can be caused by well-known neurotropic viruses such as rabies and herpesviruses. Inflammation of the spinal cord, or myelitis, which can be caused by poliovirus, is another well-described outcome of infection with a neuroinvasive virus. Furthermore, viruses have been shown capable of causing subacute CNS disorders, including the demyelinating diseases SSPE and PML, which have been demonstrated to be triggered by measles and JC viruses, respectively. These associations have fuelled research into potential links between viruses and long-term CNS complications, including neurodegenerative and neuroinflammatory diseases. Finally, the fact that viral etiologies for different neoplastic processes have been identified, provides potential links between viral infections and the development of CNS tumors. Besides the above neurotropic viruses, for which proven causal relationships with one or more CNS disorders exist, other viruses have been detected within neurological samples and may be associated with disease. This thesis assesses viral infections of the CNS, in terms of entry mechanisms and potential relationships with CNS disease. Below, these topics will be discussed, starting with viral entry into the CNS.

VIRAL ENTRY INTO THE CNS

Hematogenous transport

Viruses can enter the CNS via the circulation, a process referred to as hematogenous transport. This mode of entry likely depends upon the capacity of a given virus to elicit a viremia and effectively cross the blood-brain barrier (BBB) and/or blood-CSF barrier. These barriers are held together by tight junctions, whose permeability can be altered during an infection [1-3]. Several viruses may enter the CNS via hematogenous transport, either in the human *in vivo* situation or in animals. These viruses include morbilliviruses flaviviruses and highly prevalent respiratory viruses, as discussed in **Chapters 2, 3** and **4**, respectively. Cell entry depends upon receptor interactions and permissiveness of neural cells as addressed in **Chapter 5**. Globally, a number of hematogenous entry pathways are distinguished. These include transcellular and paracellular crossing as well as infection of BBB constituents [1-3] allowing for a more or less “direct” entry into the CNS.

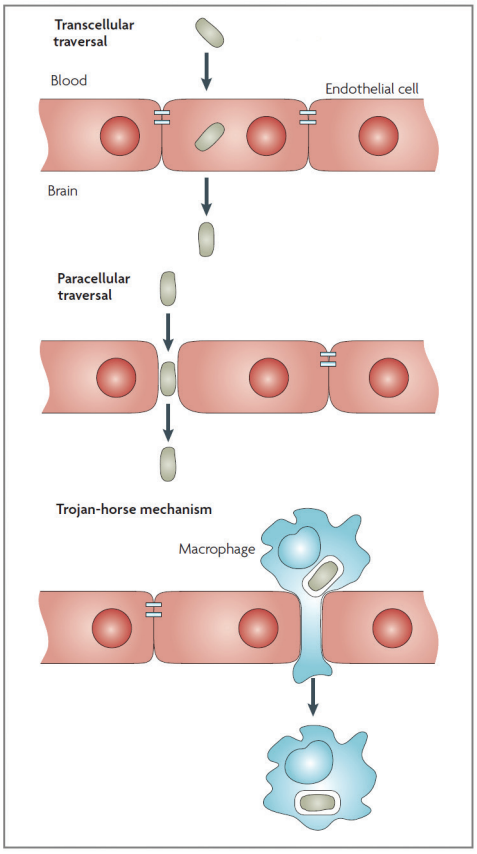

Figure 1 *Mechanisms involved in hematogenous transport into the CNS*
Apart from infection of barrier constituents, mechanisms by which viruses may enter the CNS include transcellular and paracellular spread as well as entry via "Trojan-horse" mechanisms. Adapted from [3].

In addition, trafficking of viruses within infected cells, travelling towards or being recruited by the CNS, such as immune cells, has been described, which has been termed the "Trojan-horse" mechanism *(Figure 1)*. This mechanism has been mentioned in the context of neurovirulent viruses that replicate in immune cells, including herpes, measles and retroviruses, such as HIV, simian immunodeficiency virus (SIV) and human T cell leukemia virus (HTLV) [1-3]. In this respect, the potential contribution of measles virus-infected white blood cells (WBCs) remains unelucidated, as their capacity to cross endothelial barriers is altered [2,4]. The picornavirus enterovirus 71 (EV71) and JCV can also enter the CNS via a "Trojan-horse" mechanism [1,5,6]. In the case of JCV infection, infected B cells, oligodendrocytes, and latency may, in immunosuppressed individuals, contribute to the demyelinating disease progressive multifocal leukoencephalopathy (PML) [6,7]. The case studies presented in **Chapter 7** and **Chapter 8** relate to this issue. **Chapter 7** discusses the development of an inflammatory pseudotumor in the context of an immune reconstitution inflammatory

syndrome in an individual co-infected with HIV and JCV. Here, a reactive process appears to have occurred in association with an infection with JCV, which may (initially) have spread towards the CNS within infected lymphocytes. **Chapter 8** deals with the treatment of intravascular lymphomatous cells within the CNS and may thus be of relevance with respect to (the treatment of) "Trojan-horse" entry.

Axonal transport

Axonal transport is linked to a virus' capacity to enter and travel within axons. This mode of transport has been described for the viruses discussed in **Chapters 2, 3** and **4** and also likely depends upon interactions with host cells and target tissues which, experimentally, are addressed in **Chapters 5** and **6**. Furthermore, in analogy to "Trojan-horse" entry, viruses may enter the CNS via this pathway whilst relatively "bypassing" circulatory immune responses. Trafficking associated with existing intraneural transport pathways plays an important role. In this respect, retrograde transport towards the cell body is generally mediated by dynein, or dynein-dynactin, complexes whereas anterograde transport is linked to kinesin motor proteins. These motor proteins move along microtubules that, in neurons which are "polarized" and contain axons and dendrites, have minus and plus orientations. Particularly within axons, these microtubules are oriented with the plus, or axon, end at the cell periphery and minus-end toward the cell body [1,8,9] *(Figure 2)*.

Alphaherpesviruses, poliovirus and rabies virus are well-known examples of viruses that travel intraneuronally. Viruses can enter the CNS following entry of sensory nerve endings as well as neuromuscular junctions (NMJs) [1,2,8]. Following receptor-binding, viruses may enter nerves by direct fusion with the plasma membrane or by endocytosis. Alphaherpesvirus entry occurs via direct fusion of viral envelopes with the plasma membrane after which the viral capsids may directly interact with dynein for subsequent trafficking. Most other neurotropic viruses, including non-enveloped as well as enveloped viruses, are transported within endosomes and recently, (alternative) endocytic uptake has been described for herpesviruses as well. These endosomes can be transported along existing axonal transport pathways. After retrograde transport to the cell body, viral nucleic acid genomes replicate and express messenger RNA (mRNA) and proteins. Latent infections may also occur and viral infections may have several outcomes, as will be described below (For a review of the above described (transport) processes see [1,2,8]). Anatomically, the nose and eyes are directly exposed to the external environment and the olfactory and optic nerves have direct synaptical connections to the brain, which can be relevant in terms of direct entry into the CNS whilst "bypassing" host immune responses [1,2,10,11]. Regarding influenza virus and the studies in **Chapters 5** and **6**, a recent study has demonstrated viral presence in the olfactory bulb of an immunocompromised child who succumbed as a consequence of an influenza A/H3N2 infection. Furthermore, in this report the attachment of seasonal H3N2, highly pathogenic avian influenza (HPAI) H5N1 as well as pandemic H1N1 viruses to

the apical side of the human olfactory mucosa was shown in an experimental setting [12]. Also, the postviral olfactory dysfunction (PVOD) syndrome has been documented following respiratory viral infections in humans which, among others, consists of olfactory loss as well as alterations within the olfactory epithelium, including decreased amounts of ORNs and nerve bundles [13-15] Apart from spread to the olfactory mucosa, viruses can, also upon respiratory transmission, infect the eyes and may travel within the optic nerve, rendering this another potential route of entry into the CNS [8,16]. The permissiveness of ocular cells to influenza A/H1N1pdm virus has been studied in **Chapter 6**. It is of interest that several neurodegenerative/neuroinflammatory diseases start with dysfunctioning of these olfactory (as in case of PD) and optic (as in case of MS) sensory systems, sometimes years prior to the start of other symptoms [17-19].

In vivo, immune responses are likely at play. Regarding axonal entry these may involve mucosal immunity, including type 1 IFN and IgA responses [20]. In this respect, mucosal host defense mechanisms against olfactory spread have begun to be elucidated and might include type 1 IFN responses, enzymatic reactions, transport mechanisms, cellular apoptosis and regeneration [21-24]. Immune responses may also act at the level of neuronal ganglia which, in contrast to the CNS and nerves themselves, do not have a barrier between the blood and parenchyma [2,25]. In this regard, NK cells and antigen-specific T-cells may pass fenestrated ganglionic blood vessels and act as sentinels to control infections and prevent their spread into the CNS [2,26].

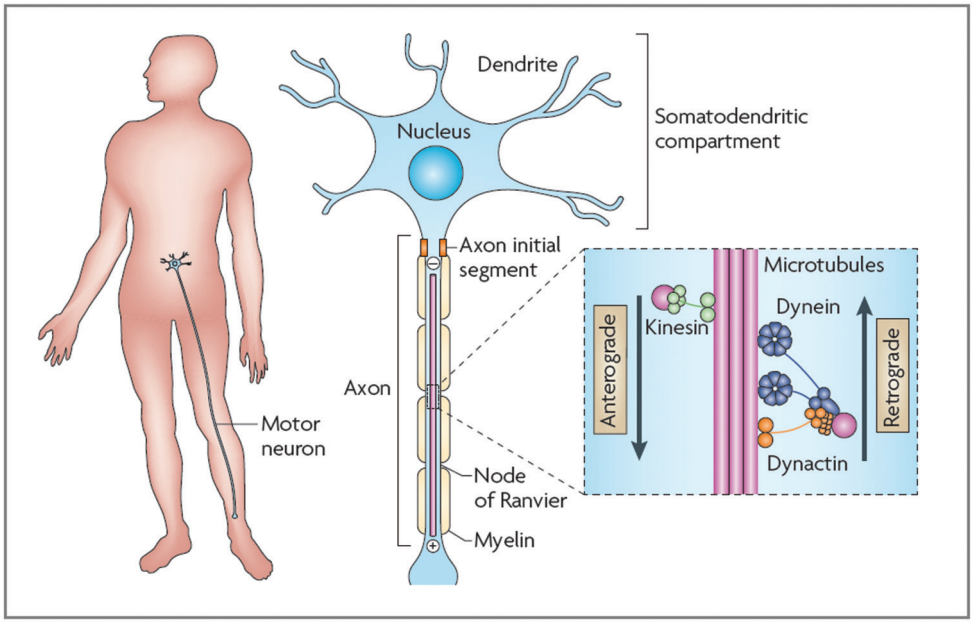

Figure 2 *Axonal transport into the CNS*
Upon entry of neural cells, axonal transport of viruses may occur along microtubules and is mediated by kinesin-coupled anterograde and dynein, or dynein-dynactin, mediated retrograde transport. Adapted from [8].

LINKS WITH CNS DISEASE

Viral neuropathology may involve several distinct processes and depend on delicate virus-host interactions.

Upon infection, viruses may have direct effects on CNS functioning. In this regard, infected neural cells, including sensory and motorneurons may succumb to the infection as a result of cytolysis or apoptosis. Also, viruses may affect infected neural cells and intracellular processes in the absence of occurrence of (immediate) cell death. The latter might certainly also be the case for viruses that have been circulating among humans for a long time. Although acute effects of viral infections on neural cells may occur, as will be described below, the avoidance of cell death might be an inherent characteristic of viral CNS infections, as will also be described below.

Besides direct virus-mediated effects, more indirect mechanisms may be involved in viral neuropathology. These often include reactions of the immune system to the infection. In terms of virus-host interactions and the effects that infections may have on the CNS, immune functioning, is of general importance. Beside adaptive (peripheral) immune responses involving T and B-cells [27], CNS innate immune responses have been characterized. Resident CNS glial cells, interacting with neurons, play important roles in these processes. Microglia are the resident brain macrophages and the archetypal cells of CNS innate immunity [28-30]. Upon infection, tissue damage or neurodegeneration, microglial reactivity, or microgliosis, may occur, which may develop into an inflammatory response, including secretion of cytokines, chemokines and radical species [28,30,31]. Although CNS innate immunity clearly relies strongly on microglia, evidence is emerging that astrocytes, which interact with microglia by means of microglial-astrocyte communication, participate in local innate immune responses as well [28,32,33]. Astrocytes display receptors involved in innate immunity such as TLRs, and under specific circumstances, such as activation or astrogliosis, might display MHC class II molecules and may participate in innate immune reactions, secreting several cytokines and chemokines [28,32,33]. Influenza, which has been studied within **Chapters 5** and **6** of this thesis, forms an interesting example of how virus-host interactions may play roles in the pathogenesis of neurological complications.

In this respect, both influenza receptor binding and membrane fusion are mediated by the viral hemagglutinin (HA) envelope protein and cleavage of this protein by cellular proteases is essential for fusion with host cell membranes. Whereas the HA of human and low pathogenic avian influenza (LPAI) viruses are believed to be cleaved by trypsin-like proteases present in the human respiratory and avian respiratory and/or intestinal tract, the HAs of HPAI viruses contain a multibasic cleavage site (MBCS) that can be cleaved by intracellular proteases present in multiple organs, such as the subtilisin-like proteases furin and proprotein convertase subtilisin-kexin type 6, which can lead to systemic infection outside the respiratory tract and increased virulence [34-36]. This broad expression pattern might explain disseminated infections, potentially following both hematogenous as well as

axonal spread, and neurological complications reported upon infection with avian viruses. ORNs, which form part of the respiratory tract, may express trypsin-like proteases. Observations that neural cells, as well as olfactory organs-for which studies on animal tissue have been described-, contain trypsin-like proteases might explain the finding of human influenza viruses in the human CNS as well as potentially (part of) reported neurological complications for these viruses, as well. Maybe these latter viruses, as also evidenced by studies described within this thesis, at least can spread via axonal routes. Regarding potential effects following CNS entry, *in vitro* studies have demonstrated that influenza viruses can productively infect human neuronal and astrocytic cells [37,38]. The replication competences for human and avian viruses here, were similar [37]. Furthermore, distinct cytopathic effects and induction of cytokine responses were observed [37]. H5N1 viruses induced pronounced cytopathic effects and induced high cytokine (IL-6 and TNF-α) responses in infected human cells [37].

These observations indicate that both viral as well as host factors may play roles in the pathogenesis of CNS pathology. Against the background of this notion, potential relationships between viruses and CNS disorders will be described in detail below.

Acute CNS diseases

As mentioned above, direct interactions of viruses, or viral proteins, with neural cells have been described. Furthermore, (potentially peripheral) immune responses might play a role in the development of CNS disease as they, for instance via the release of cytokines, can augment barrier permeabilities (as described above) and lead to CNS dysfunctioning or encephalopathy [2,27,39-45]. Regarding direct virus-mediated effects, neurotropic viruses can induce cytolysis or apoptosis in infected cells [46,47]. In neurons, this process depends upon neuronal maturity (with an increased susceptibility of immature neurons); viral virulence (neurovirulent viruses being able to overcome anti-apoptotic defenses of mature neurons), and host immune responses, and is caspase-dependent [47].

As mentioned, several viruses are capable of causing acute CNS disorders including encephalitis and myelitis. Potentially associated pathogenic mechanisms have been discussed in **Chapter 3**. Suggested pathogenic mechanisms by which encephalitic viruses such as JEV and TBEV cause CNS disease indeed include both cell death as well as bystander damage. Also, distinct host factors including CCR5, OAS and TLR3 mutations have been suggested to play a role. Furthermore, WNV infects spinal cord motor neurons and causes acute flaccid paralysis/poliomyelitis. Here an axonal entry mechanism may play a role, as indicated by animal experiments. Furthermore, mechanisms involved in the pathogenesis of WNV-associated CNS disease have also been suggested to include apoptosis, bystander

damage and host factors including CCR5 and OAS mutations, TLR3 dysregulation, and CD4 and CD8 T-cell defects. Experimentally, potential pathogenic mechanisms involved in viral CNS infections have been addressed in **Chapters 5** and **6**, dealing with CNS entry routes and permissiveness of neuronal cells.

Subacute CNS diseases

Several viruses have been shown to be capable of causing subacute CNS diseases. Examples include the causative link between measles virus and SSPE which has been discussed in **Chapter 2**. Suggested mechanisms that play a role in the pathogenesis of SSPE involve viral persistence, (immune) cell-mediated damage and lysing CSF antibodies. JCV has been demonstrated to cause PML as discussed in **Chapter 7**, which describes a potential immunological "re-targeting" of JCV in the context of an immune reconstitution syndrome, following treatment of an HIV-infected individual with HAART.

Neuroinflammatory/neurodegenerative diseases

Several of the viruses discussed in this thesis have been linked to major "hallmarks" of neurodegenerative and/or neuroinflammatory diseases, both in terms of pathology and symptomatology. These hallmarks include demyelination, anterior horn pathology, parkinsonism and protein aggregation. Additional hallmarks, including inflammation, cell loss/apoptosis and gliosis, have been discussed in the above sections and may play a role in neurodegenerative/neuroinflammatory diseases as well. The time course in which eventual CNS pathology develops may then depend upon the (acute or chronic) nature of virus-host interactions, as will be discussed below.

Possibly involved pathogenic mechanisms that underly the occurrence of these hallmarks likely include affection of specific cell types as experimentally assessed in **Chapters 5** and **6**, and intracellular processes. In this regard, as discussed in the introduction, herpesviruses can infect the temporal lobes and interact with amyloid precursor protein (APP) during their cellular transport. As also discussed in **Chapter 2** and **7**, morbilliviruses and JC virus affect cells involved in myelin synthesis and homeostasis, such as oligodendrocytes and astrocytes and are associated with demyelination. As discussed in **Chapter 3**, WNV affects anterior horns and is associated with acute flaccid paralysis (AFP), whilst JEV infects the midbrain, which is affected in PD, and is associated with parkinsonism. Furthermore, as discussed in **Chapters 4, 5** and **6**, influenza has been shown capable of olfactory transport, the olfactory system having been described to be involved in PD, and to induce neuroinflammatory/neurodegenerative features, including protein aggregation, in animal experiments.

Especially regarding long-term neurodegenerative/neuroinflammatory diseases, pathogenic mechanisms may include multiple "subclinical infections" throughout a lifetime, in such a sense that viruses may enter the CNS via axonal, potentially olfactory, transport

and thus travel towards the CNS within neurons whilst relatively "bypassing" circulatory immune responses. These entry routes have been discussed in several chapters of this thesis, including **Chapter 4, 5** and **6**, and the above section on viral entry. In an analogous fashion, they may enter within lymphomatous cells, which are discussed in **Chapter 8** and the above section.

Potentially in combination with this means of spread, the notion of viral latency may play a pivotal role with respect to the assessment of potential associations with "long-term" neurodegenerative/neuroinflammatory complications as this may be an inherently "favourable" outcome of neurological infections and viruses may remain latently present within neuronal cell bodies of either the peripheral nervous system (PNS) and/or the CNS. Latency of herpesvirus infections has been well-studied and has also been reported or suggested for morbilliviruses, including measles virus, flaviviruses, several highly prevalent respiratory viruses which could be retrieved from CNS tissue samples following prolonged incubation times, and JCV which are discussed in **Chapters 2, 3, 4** and **7**. In case of the well-documented herpesvirus infections, quiescent virus genomes are often not integrated into the host genome and either exist as nonreplicating histone-covered molecules or as replicating extra "chromosomes" [1,48]. After latency has established, its maintenance requires ongoing action of glial and virus-specific CD8+ T-cells that suppress lytic gene expression [1,49]. Occasionally, infections can reactivate, potentially during (relative) immune suppression [1,8]. In these respects, neurons, which can tolerate high viral inputs and gene expression controlled by an immune response that is typically non-cytolytic, as well as neurotropic viruses, have both evolved to avoid cell death, which may be related to the irreplaceability of neurons [1,27]. Indeed, mutual prosurvival strategies may allow neurons to survive both latent as well as reactivated infections [1]. These inherent characteristics of virus-host interactions within the CNS make links with "slow" neurodegenerative and neuroinflammatory diseases conceptually possible and of interest to study.

Because of their capacity to travel "subclinically" and establish latency, within neurons, the question is how often viral infection of the CNS in fact could occur and lead to brain dysfunction [12,17,50-52]. Pathogenically, also when neural cells are not affected in an acute manner, viruses can interact with existing cellular processes including those involved in transport, metabolism, degradation and recycling or autophagy, signaling, and synaptic transmission [1,2,8,40-42,46]. As also mentioned, these interactions can involve distinct proteins such as amyloid precursor protein (APP) [40,41,53-55]. Apart from their potency to induce general neuroinflammatory and neurodegenerative tissue responses, these processes could induce partial structural alterations including axonal damage and degeneration [40-42,46]. Experimental studies have suggested long-term pathological effects can eventually take place in the context of potentially latently present and asymptomatically reactivating infections, potentially allowing for remitting pathologies [40-42,56,57].

In mechanistical terms, apart from viruses as a whole, individual viral proteins can play roles in pathological processes [39,46]. With respect to persistent infections, there can, in this regard, continue to be a restricted expression of viral proteins when a nonreplicative state of the genome is maintained [39,58]. Furthermore, in analogy to the above discussed encephalopathic mechanisms, viral presence within the CNS or prolonged exposure to viral proteins may not be necessary to exert pathological effects and transient exposures may be sufficient to trigger a cascade of events resulting in neuropathology, i.e. the so-called "hit and run phenomenon" in which several cell types and feedback loops may be involved [39]. Also, infections occasionally trigger autoimmune responses, which may occur via several mechanisms [39,59]. Upon cellular egress, enveloped viruses may carry antigens from the host cell membrane that become incorporated into the viral envelope [39]. Viral proteins may furthermore display similarities to host proteins, causing these latter proteins to be affected by immune responses via the mechanism of "molecular mimicry", although concepts in this respect might be developing [39,60-62].

In general, it is of importance that all the above processes, and the potential development of neurodegenerative/neuroinflammatory pathologies, might occur in interaction with other factors, including oxidative stress, ageing, concomitant metabolic diseases and host genetics [40-42,57]. These factors may be interrelated with antiviral immune responses, for instance through the mechanism of immunosenescence [63]. As also mentioned in the Introduction, genetic as well as sporadic forms, or factors, have been identified for CNS diseases. Possible long-term interactions of viruses with the CNS and their potential role in the development of neuroinflammatory/neurodegenerative disorders, warrant further study.

CNS tumors

As described within the introduction, viral etiologies of tumors have been demonstrated. Studied viruses and links have included polyoma viruses. In **Chapter 7** of the thesis an inflammatory pseudotumor is described which consisted of giant cells with pleomorphic hyperchromatic nuclei, often multiple, surrounded by a dense infiltrate of plasma cells. These cells were GFAP positive, demonstrated diffuse nuclear reactivity for p53 antigen and a high MIB-1 (Ki-67) index. This combination and dense inflammatory infiltrates resulted in the formation of a JCV-associated pseudotumor.

Studies on viruses and links with the development of CNS tumors have also included herpesviruses. In this respect, **Chapter 8** describes the development of IVL of the brain in an individual diagnosed with MDS. For both these disorders, links with infectious agents have been reported, which include associations with herpesviruses in case of IVL. The question, in this respect, remains whether lymphocytes harbouring oncogenic viruses can enter the brain parenchyma and induce parenchymatous tumors, via "Trojan-horse" entry as described within this thesis.

FUTURE PERSPECTIVES

Central to the topics discussed in this thesis, developments in the fields of molecular detection and imaging will be crucial in the further identification and characterization of viruses that can infect the CNS and the establishment of associations between CNS conditions and viral infection. Here screening studies are of utmost importance. These could include viral detection studies using biomaterials, such as brain tissue and CSF samples, assessing the prevalence of distinct viruses in a (specific) population and/or affected brain regions and cell types. Also, imaging will be highly important. As discussed in the Introduction, in this respect, using distinct tags or markers and real-time techniques, specific infectious processes might be visualized, for example during a MS relapse or ongoing neurodegenerative process.

Concerning therapeutic options, (further) development of virus-specific therapies that cross or bypass the BBB and blood-CSF barrier are of interest. Size, lipophilicity, plasma protein binding characteristics, distribution volumes, and affinities for efflux pumps present at the blood-brain and/or blood-CSF barriers, are important determinants of therapeutic efficacies and of the capacity of drugs to reach the CNS [64]. Apart from hematogenous administration, intraneuronal delivery strategies may be an interesting therapeutic route. Intra-axonally, also intra-olfactory, delivery strategies have been suggested and developed and it is of interest to study these [65-72] *(Figure 3)*.

In terms of prevention, interventions that act before axonal entry takes place, e.g. at the level of the (nasal) mucosa are of interest. In this respect, it has been demonstrated that, in animals, intranasal influenza immunization, followed by homologous viral challenge, suppressed the levels of viral RNA 6 in the olfactory bulb 6 days after viral challenge, as well as the acute phase response [73].

While the above therapies may be virus- and CNS-specific, personalized strategies at the level of infected individuals can also be (further) developed, likely depending on the aforementioned developments in the fields of detection methods and viral and human metagenomics [74]. Developments in these fields provide clues about when and whom to treat, as potentially not all viruses, in all hosts, are always disease-causing. It can, in this respect, be of relevance to further or even prior to the start of treatment, assess both (individual) immune and microbial profiles, as mitigating immune responses can have clinical consequences, as exemplified by cases of JCV-related PML occurring in the setting of treatment with immunomodulatory therapies. In analogy, it may, in certain and carefully selected instances and individuals, be an option to (empirically) treat with antiviral compounds. This might have a therapeutic effect and aid in the specification of disease etiologies. In this respect, therapy with herpes antivirals in the context of patients with mild AD have been speculated upon [40]. Also, two clinical trials were started, involving treatment of glioblastoma (GBM) patients with valganciclovir and CMV pp-65-modified dendritic cell vaccines [75]. Furthermore, a study using the protease inhibitor indinavir was set-up by the

US ALS society [76]. Toxicity discouraged further trials with the latter drug [77]. Although this is a possible outcome of such studies, it will be of interest to see what place there can be for (empirical) antiviral treatments, notably including co-administration of antivirals next to immunomodulators, in case of CNS disorders of potential (partial) viral origin, also since some of these disorders are being treated with immunomodulating therapies against the background of a CNS virome harbouring potentially latent infections.

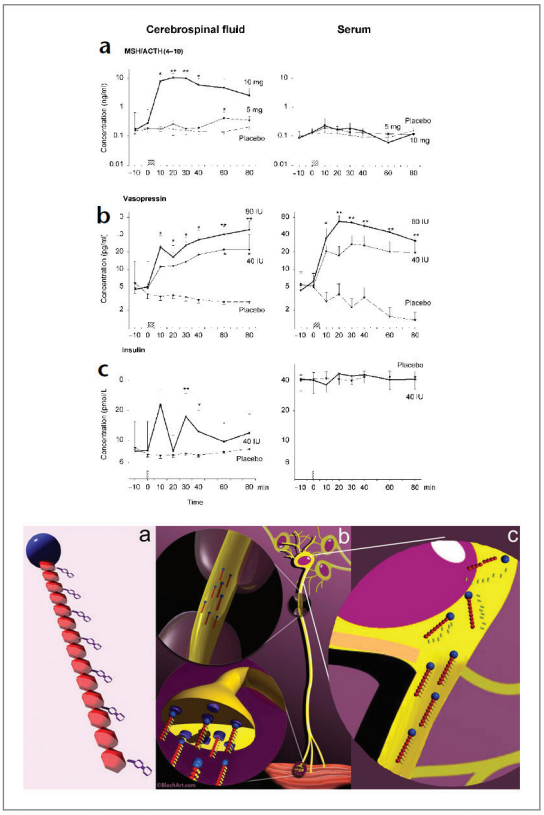

Figure 3 *Targeted drug delivery to the CNS*
Top: Concentrations of several neuropeptides in CSF and serum 10 mins before to 80 mins following intranasal administration in humans (a: melanocortin(4-10) (MSH/ACTH(4-10); b: vasopressin; c: insulin). Adapted from [65]. *Bottom: Schematic model of a tripartite vehicle for axonal transport drug delivery which has been tested in animal models of infection (a: the drug delivery vehicle includes a targeting element-the axonal transport facilitator/ATF (blue), and a polymer (red repeating units) that carry drug molecules (purple); b: intraneural transport takes place; c: late during transport and on arrival in the soma, lytic processes release the active drug molecule by breakdown of linker components). Adapted from* [68].

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